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Effect of Microbial Inoculants on the Indigenous Actinobacterial Endophyte Population in the Roots of Wheat as Determined by Terminal Restriction Fragment Length Polymorphism

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The effect of single actinobacterial endophyte seed inoculants and a mixed microbial soil inoculant on the indigenous endophytic actinobacterial population in wheat roots was investigated by using the molecular technique terminal restriction fragment length polymorphism (T-RFLP). Wheat was cultivated either from seeds coated with the spores of single pure actinobacterial endophytes of *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *Nocardioides albus* EN46 or from untreated seeds sown in soil with and without a commercial mixed microbial soil inoculant. The endophytic actinobacterial population within the roots of 6-week-old wheat plants was assessed by T-RFLP. Colonization of the wheat roots by the inoculated actinobacterial endophytes was detected by T-RFLP, as were 28 to 42 indigenous actinobacterial genera present in the inoculated and uninoculated plants. The presence of the commercial mixed inoculant in the soil reduced the endophytic actinobacterial diversity from 40 genera to 21 genera and reduced the detectable root colonization by approximately half. The results indicate that the addition of a nonadapted microbial inoculum to the soil disrupted the natural actinobacterial endophyte population, reducing diversity and colonization levels. This was in contrast to the addition of a single actinobacterial endophyte to the wheat plant, where the increase in colonization level could be confirmed even though the indigenous endophyte population was not adversely affected.

Cereal production is an important source of food grains and is expected to play a key role in meeting the needs of the world population, which is growing by 160 persons per min (11). In order to achieve an increase in agricultural productivity in a sustainable manner, there will be increased reliance on manipulation of microorganisms that are beneficial to soil and plant health.

A major factor influencing plant growth and health is the microbial population living both in the rhizosphere and as endophytes within healthy plant tissue. A portion of these microorganisms possess the ability to suppress disease and/or promote growth and are termed plant growth-promoting rhizobacteria (PGPR). PGPR were first used to improve crop fertility by increasing the amount of nitrogen available to the plant. The most efficient nitrogen-fixing strains belong to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Allorhizobium* (3). Some success has been achieved in controlling crop pathogens and promoting plant growth by supplementing the crop soil with these PGPR and other biocontrol microbial inocula (3). Microbial biocontrol agents have been shown to inhibit soil-borne pathogens such as *Fusarium oxysporum*, *Gaeumannomyces graminis*, *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani*, and *Verticillium* spp. (7, 12, 14, 18, 22, 29, 30). However, a large number of biocontrol agents fail to be effective due to the difficulty of manipulating the highly complex rhizosphere environment. Endophytic microorganisms may offer an advantage in terms of efficiency of colonization leading to improved effectiveness. There appears to be a close relationship between the rhizosphere and endophyte populations. As demonstrated in some cases, when complementary crops were grown in rotation they shared 70% of the same species of endophytic bacteria (25). Recently, it was shown that a *Streptomyces lydicus* strain previously isolated from rhizosphere soil could colonize root surfaces and cause physiological changes to the nodules of pea plants (26).

Endophytic bacteria have been isolated from a variety of plants, including tomato (*Solanum lycopersicum* L.) (19), potato (*Solanum tuberosum* L.) (8, 24), wheat (*Triticum aestivum* L.) (6, 9, 22), sweet corn (*Zea mays* L.) (17), cotton (*Gossypium hirsutum* L.) (17), oilseed rape (*Brassica napus* L.) (9, 19), and citrus plants (1, 2).

Endophytic bacteria have the ability to promote growth and inhibit plant disease, and as they are in intimate contact with the plant they are an attractive choice as biological control agents. For example, Sturz et al. (24) found that 61 of 192 endophytic bacterial isolates from potato stem tissues were effective biocontrol agents against *Clavibacter michiganensis* subsp. *sepedonicus*. In oak, endophytic bacteria biologically active against the oak wilt pathogen *Ceratocystis fagacearum* have been isolated (4).

A number of the biologically active endophytes and root-colonizing microorganisms that have been isolated or detected belong to the actinobacterial phylum, specifically the genus *Streptomyces* (6, 8, 21, 23, 26, 29). The first actinobacterial endophyte isolated, belonging to the genus *Frankia*, is a nitrogen-fixing actinobacterium that forms actinorhizae with eight

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families of angiosperms (20). A number of endophytic actinobacteria were previously isolated by culture-dependent methods, with the major genera being *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardioides* (6). A number of these isolates were capable of suppressing fungal pathogens of wheat in vitro and in planta, including *Rhizoctonia solani*, *Pythium* spp., and *Gaeumannomyces graminis* var *tritici*, indicating their potential use as biocontrol agents (7).

It is well known that the addition of nonindigenous microbes to soil can affect the indigenous rhizosphere population (28). However, the effect of microbial inoculants on the endophytic population is unknown. In this study the effect of a commercial mixed microbial inoculant, NutriLife 4/20 (NutriTech, Eumundi, Australia), and single actinobacterial endophytic inoculants on the indigenous actinobacterial endophyte population of wheat was investigated. The culture-independent technique terminal restriction fragment length polymorphism (T-RFLP) (10, 15) was used in conjunction with a semiquantitative analysis protocol (5).

MATERIALS AND METHODS

Actinobacterial cultures. Actinobacterial cultures used in this study were *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *Nocardioides albus* EN46. Actinobacterial endophytes were isolated from healthy wheat roots previously (6). Cultures were maintained on mannitol soy (MS) agar, oatmeal agar (OA), or half-strength potato dextrose agar (PDA).

Growth of actinobacteria and harvesting of spores. Actinobacterial cultures of *Streptomyces* sp. strain EN27 and *N. albus* EN46 were grown on MS agar and PDA, respectively, whereas *Microbispora* sp. strain EN2 was grown on OA. Plates were incubated at 27°C for 3 to 10 days until abundant sporulation had occurred. Spores were harvested from the plate with a sterile loop and were suspended in 2 ml of sterile water. Each suspension contained approximately 10^{12} spores/ml.

Coating wheat seeds with actinobacterial spore inoculum. Approximately 40 wheat seeds (cultivar Krichauff) were placed in a sterile petri dish. Two milliliters of the actinobacterial spore suspension was poured over the seeds and mixed. The petri dish was left in the laminar flow cabinet overnight to evaporate the water and coat the spores onto the seeds.

Wheat cultivation. Seeds coated with the actinobacterial spores were cultivated in pots (100 mm height, 50 mm diameter) containing soil obtained from a wheat field in Haslam, located on the Eyre Peninsula, South Australia. Untreated seed was cultivated and used as a control sample. Investigation of the effect of a mixed microbial soil inoculant was performed by cultivating wheat seeds (cv. Krichauff) in soil obtained from Swedes Flat, located in the southeast of South Australia. Two soil samples were obtained from this site, one of which was treated with the commercial inoculant (NutriLife 4/20; NutriTech). Nutrilife 4/20, available as granules, contained 20 bacterial strains, including *Streptomyces albidoflavus* and *S. cellulosae*, and four fungal species (NutriTech, personal communication). The granules were mixed with the NutriTech nutrient formulation in water according to the manufacturer's instructions (www.nutri-tech.com.au) for 24 h before use. One-hundred liters of this microbial suspension was added to soil at a rate of 100 liters per hectare. The soil was collected for immediate use in pot trials. For all plant treatments three seeds were sown per pot and three replicate pots were sown. All plants were cultivated in a glasshouse with watering as required. Plants were harvested 6 weeks after germination.

DNA extraction from actinobacterial cells. Total genomic DNA was extracted from the actinobacterial spores by using a modified cetyltrimethylammonium bromide/NaCl protocol (13) as described previously (5).

Extraction of endophytic bacterial DNA from the roots of wheat. Endophytic bacterial DNA was extracted from the roots of 6-week-old wheat plants. The root material was first surface sterilized by using an ethanol-sodium hypochlorite procedure, and the endophytic bacterial DNA was extracted as described previously (5). Three replicate root samples were used for each treatment.

T-RFLP analysis of 16S rRNA gene amplification products. Partial 16S rRNA gene sequences were amplified from the endophytic DNA using the actinobacteria-biased primers 243f (5′ GGA TGA GCC CGC CGC CTA 3′) (10) and 5′TET (6-carboxy-2′,4,7,7′-tetrachlorofluorescein)-labeled 1492r (5′ TA CGG GTA CCT TGT TAC GAC TT 3′) (27) prepared by GeneWorks, Adelaide, Australia. Two amplifications per sample were carried out according to Conn and Franco (5), resulting in six replicate PCR products for each treatment. Single restriction digestions of each of the 16S rRNA PCR products were performed with HinfI, HhaI, and MboI (Promega), using 10 μl of the PCR mixture for 16 to 18 h to achieve complete digestion, and then the digests were stored at −20°C. The size of the terminal 16S rRNA gene fragments present in the restriction digestions were determined on an automated Applied Biosystems 373 DNA sequencer, Stretch, using 1 μl of the restriction digest. Data was analyzed using the GeneScan Analysis program V.3.1.2 (Applied Biosystems). The terminal restriction fragment (TRF) sizes present for each restriction enzyme were determined from the GeneScan data.

Statistical and data analysis. Semiquantitative analysis of the T-RFLP data was carried out as described in Conn and Franco (5).

TABLE 1. Fragment sizes of *Microbispora* sp. EN2, *Streptomyces* sp. EN27, and *N. albus* EN46 16S rRNA gene sequence digested with HinfI, HhaI, and MboI

Species (GenBank accession no.) and enzyme[a]	Predicted fragment size (bp)	Actual fragment size (bp)
EN2 (AY148073)		
HinfI	179, 232–235	175.3
HhaI	470–472, 490	418.7
MboI	162, 215–218	162.4
EN27 (AY14805)		
HinfI	236–240	240.6
HhaI	419	419.7
MboI	158	163.3
EN46 (AY148081)		
HinfI	175	178.5
HhaI	410	410.5
MboI	157	162.7

[a] EN2, *Microbispora* sp. strain EN2; EN27, *Streptomyces* sp. strain EN27; EN46, *N. albus* EN46.

RESULTS

Detection of introduced endophytes in the roots of wheat by T-RFLP. The pure cultures of the three actinobacterial endophytes, *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *N. albus* EN46, investigated in the study were analyzed by the T-RFLP technique to determine the unique TRFs obtained with the restriction enzymes HinfI, HhaI, and MboI. Table 1 shows the fragment sizes obtained from the digestion of the 16S rRNA gene sequences from each endophyte used in this study and for each restriction enzyme. There were unique fragment sizes for each of the added endophytes and restriction enzyme, which enabled the introduced endophytes to be detected in the roots of wheat by corresponding the fragment sizes and peak areas to the specific inoculant.

The endophytic bacterial DNA isolated from the roots of endophyte-inoculated and uninoculated wheat plants at 6 weeks of growth was subjected to the T-RFLP procedure. A representative T-RFLP profile obtained with HinfI for one of these plant treatments is shown in Fig. 1; the annotated peaks indicate the fragment corresponding to the introduced actinobacterial endophyte. Table 2 shows the three specific TRFs corresponding to the inoculated endophytes and the minimum and maximum abundance percentages of the peak areas (not corrected). The minimum and maximum abundance percentages were calculated by dividing the area of the peak of interest by the total peak areas for that particular T-RFLP profile. The

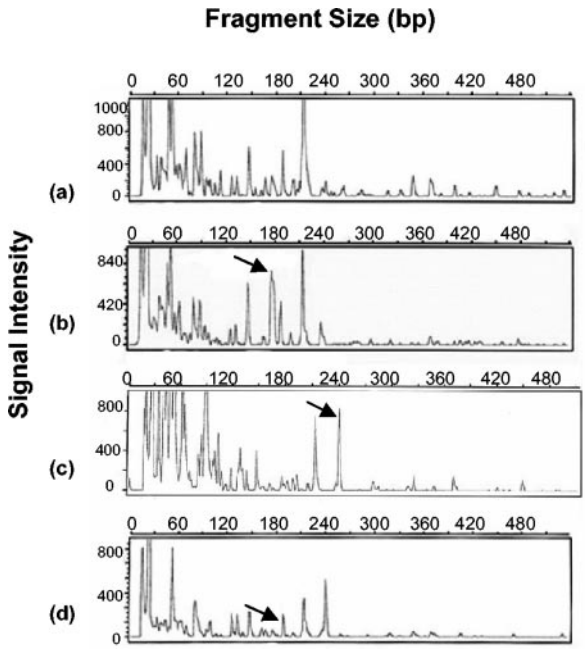

FIG. 1. T-RFLP HinfI profiles for the roots of wheat grown from uninoculated seed (a), *Microbispora* sp. EN2-inoculated seed (b), *Streptomyces* sp. EN27-inoculated seed (c), and *N. albus* EN46-inoculated seed (d). The highlighted peaks correspond to the specific fragment of the specific actinobacterial endophyte inoculated onto the seed.

percentages were calculated for each replicate in the plant group, as the peak areas are specific for each T-RFLP profile. The minimum and maximum abundance percentages were determined for each specific TRF from the six replicates.

From these results it was observed that the maximum abundance percentage for the MboI fragment of *Microbispora* sp. strain EN2 increased by 1.1-fold, whereas the HinfI and HhaI fragments increased by approximately two- and threefold, respectively, indicating that colonization has occurred. The specific 241-bp HinfI fragment corresponding to *Streptomyces* sp. strain EN27 was not present in the uninoculated control, and the HhaI-specific fragment increased by twofold in terms of maximum abundance percentage. This provided a good indication that colonization had occurred. Analysis of the specific fragments for *N. albus* EN46 showed that the HhaI and HinfI peaks decreased, while the MboI-specific fragment increased 2.2-fold in terms of maximum abundance percentage.

TABLE 2. Comparison of the minimum and maximum fragment percentages obtained from T-RELP profiles of endophytic bacterial DNA isolated from the roots of wheat inoculated with an endophyte to those of an uninoculated plant ($n = 6$)[a]

Restriction enzyme and inoculant	Fragment of interest (bp)	Fragment % for inoculated wheat		Fragment % for uninoculated wheat	
		Min	Max	Min	Max
EN2					
HinfI	176	0.96	6.17	0.69	3.33
HhaI	420	2.98	10.94	1.69	3.33
MboI	162	1.58	11.08	3.62	10.13
EN27					
HinfI	241	1.26	3.55	ND	ND
HhaI	420	2.77	5.80	1.69	2.96
MboI	163	4.19	7.06	3.62	10.13
EN46					
HinfI	179	0.58	5.41	0.38	5.78
HhaI	411	1.02	9.45	4.61	14.57
MboI	162	1.87	23.19	3.62	10.13

[a] EN2, *Microbispora* sp. strain EN2; EN27, *Streptomyces* sp. strain EN27; EN46, *N. albus* EN46. ND, not determined.

Effect of introducing actinobacterial endophytes on the indigenous endophytic population of wheat roots. As the T-RFLP technique was able to detect colonization of the introduced endophytes, the effect of these inoculants on the indigenous endophytic actinobacterial population was determined by T-RFLP and was compared to that of the uninoculated control. The actinobacterial 16S rRNA gene was amplified from the endophytic bacterial DNA extracted from the roots of wheat grown from inoculated or uninoculated seeds. The TRF sizes obtained in at least four of the six replicates with the restriction enzymes HinfI, HhaI, and MboI are shown in Table 3. These triple TRFs were corresponded to bacterial species by using the TAP-T-RFLP database (5, 16). Among the four different plant treatments, a total of 58 genera were identified. The 49 genera which had a maximum abundance percentage of more than 0.6% in all treatments are listed in Table 4. Thirty-six genera were present in all treatments, and 45 genera were present in three of the four treatments.

The effect of a commercial mixed microbial soil inoculant on the endophytic actinobacterial population in the roots of wheat. The effect of the commercial mixed microbial inoculant on the endophytic actinobacterial population in the roots of wheat was determined by T-RFLP analysis of the 16S rRNA gene sequence. Figure 2 shows the GeneScan T-RFLP profile of the actinobacterial 16S rRNA gene TRFs amplified from the roots of wheat grown in inoculated and uninoculated soil and digested with HinfI. The actinobacterial 16S rRNA gene terminal fragment sizes obtained with HinfI, HhaI, and MboI that were present in at least four of the six replicates are shown in Table 5. These fragments were corresponded to bacterial species by using the TAP T-RFLP database (16). The maximum abundance percentage of each actinobacterial genus present and the percentage of reduction of genera in the inoculated soil are shown in Table 6. Forty actinobacterial genera were detected in the untreated soil, but in the presence of added NutriLife 4/20 inoculum the number of detectable genera was reduced to 21. The level of relative colonization was also lowered, between a range of 14 to 86%, compared to that of plants grown in the uninoculated soil.

DISCUSSION

There is an emerging trend to replace chemical herbicides, fungicides, and fertilizers with microbial inoculants due to environmental and health concerns. In 2001, 65% of the nitrogen supply to crops originated from PGPR. In addition, a number of PGPR are used as biological control agents for the suppression of soil-borne diseases (1, 21). It is well known that the addition of nonindigenous microbes to the soil can affect the

TABLE 3. 16S rRNA gene sequence fragment sizes (in base pairs) obtained with the restriction enzymes HinfI, Hha, and MboI for the roots of wheat inoculated with (*Microbispora* sp. EN2 [2], *Streptomyces* sp. EN27 [27], *N. albus* EN46 [46]) or without (UN) an endophyte and grown for 6 weeks in soil obtained from Haslam ($n = 6$)

HinfI	UN	2	27	46	HhaI	UN	2	27	46	MboI	UN	2	27	46
34	+	+	+	+	34	−	−	−	+	34	+	+	+	−
39	+	+	+	+	39	+	+	+	+	36	−	+	+	+
45	+	+	+	+	42	+	−	−	−	39	+	+	+	+
49	+	+	−	+	45	+	+	+	+	45	+	+	+	+
53	+	+	+	+	49	+	+	+	+	49	+	+	+	+
58	+	+	+	+	53	+	+	+	+	53	+	+	+	+
62	+	+	+	+	58	+	+	+	+	58	+	+	+	+
63	+	+	+	+	63	+	+	+	+	61	+	+	+	+
67	−	−	+	−	70	+	+	+	+	64	+	+	+	+
70	+	+	+	+	74	−	+	−	−	67	−	−	+	−
76	−	+	+	−	77	−	−	+	−	70	+	+	+	+
81	+	+	+	+	81	+	+	+	+	77	−	−	+	−
84	+	+	+	+	83	+	+	+	+	81	+	+	+	+
88	+	+	+	+	88	+	+	+	+	82	+	+	+	+
91	+	+	+	+	91	+	+	−	−	84	+	+	+	+
95	+	+	+	+	95	+	+	+	+	88	+	+	+	+
97	+	+	+	+	97	+	+	+	−	92	+	+	+	+
100	+	+	+	+	100	−	+	+	+	95	+	+	+	+
112	+	+	+	−	106	−	+	−	+	97	+	+	+	+
126	+	+	+	+	120	−	−	+	−	100	−	+	+	−
132	+	+	+	+	112	+	−	−	−	112	+	+	+	−
147	+	+	+	+	126	+	+	+	+	132	+	−	−	+
163	+	+	+	+	132	+	+	+	+	134	+	−	+	+
167	+	+	+	+	147	+	+	+	+	153	+	−	+	+
176	+	+	+	+	162	+	−	+	−	158	+	+	+	+
178	+	+	+	+	175	+	+	+	+	162	+	+	+	+
180	+	+	+	+	190	+	+	+	+	163	−	+	+	+
189	+	+	+	+	200	−	+	−	−	166	−	−	+	+
201	+	+	+	+	214	+	+	+	+	173	+	+	+	+
214	+	+	−	+	227	−	−	+	−	175	+	+	−	+
219	+	−	−	−	279	+	+	−	−	183	+	−	+	−
232	−	−	+	−	332	+	−	−	−	214	+	−	+	−
237	+	+	+	−	348	+	+	+	−	218	+	−	+	−
241	−	+	+	+	369	+	−	+	−	284	+	−	+	−
348	+	+	−	+	387	+	+	+	−	287	+	−	−	−
354	+	−	−	−	398	+	+	+	−					
369	+	+	+	+	412	+	+	+	+					
					413	+	+	+	+					
					416	+	−	−	+					
					420	+	+	+	+					
					422	+	−	+	+					
					470	+	−	−	+					

TABLE 4. Maximum abundance percentage of actinobacterial genera in the roots of wheat grown from seed inoculated with endophytes (*Microbispora* sp. EN2 [EN2], *Streptomyces* sp. EN27 [EN27], *N. albus* EN46 [EN46]) and uninoculated seed for 6 weeks in soil obtained from Haslam (genera with a maximum percent under 0.6% in all samples were omitted) ($n = 6$)

Genus	Uninoculated	EN2	EN27	EN46
Kribbella	13.72	13.21	13.68	7.19
Streptomyces	12.15	18.37	20.12	30.66
Bifidobacterium	6.05	11.92	3.98	15.96
Arthrobacter	5.26	5.79	2.73	3.08
Nocardia	4.00	3.34	0.39	0.87
Actinoplanes	2.92	0.6	0.00	0.00
Rhodococcus	2.81	7.03	1.16	3.63
Kineococcus-like bacterium	2.54	3.04	0.28	0.86
Nocardioides	2.53	3.18	1.04	4.18
Rubrobacter	2.46	2.08	0.33	1.07
Geodermatophilus	2.27	4.29	1.13	2.2
Mycobacterium	2.27	3.45	1.00	3.31
Microbispora	2.26	2.34	1.18	1.66
Brevibacterium	2.16	4.38	0.56	1.93
Thermomonospora	2.10	4.42	1.68	3.4
Saccharomonospora	1.73	4.01	0.66	0.32
Corynebacterium	1.62	3.02	0.53	1.41
Frankia	1.62	3.28	0.56	1.61
Kitasatospora	1.62	1.37	0.14	0.43
Microbacterium	1.62	1.92	0.64	1.53
Actinosynnema	1.46	0.30	0.00	0.00
Spirilliplanes	1.46	0.30	0.00	0.00
Streptoalloteichus	1.46	0.30	0.00	0.00
Agromyces	1.08	0.28	0.25	1.10
Catellatospora	1.08	1.64	0.28	0.86
Cellulomonas	1.08	0.27	0.14	0.43
Gordonia	1.08	1.65	0.91	1.10
Lechevalieria	1.08	1.64	0.28	0.86
Lentzea	1.08	1.37	0.28	0.75
Micrococcus	1.08	1.64	0.28	2.09
Pimelobacter	1.08	0.00	0.24	0.71
Sarraceniospora	1.08	1.64	0.28	0.86
Terrabacter	1.08	0.54	0.28	0.86
Actinomyces	0.83	1.37	0.14	0.43
Saccharothrix	0.65	1.27	0.38	0.00
Thermocrispum	0.65	2.23	0.38	6.07
Aeromicrobium	0.54	1.37	0.14	0.43
Candidatus Microthrix	0.54	1.37	0.14	0.43
Cellulosimicrobium	0.54	1.37	0.14	0.86
Pilimelia	0.54	1.64	0.39	0.98
Prauseria	0.54	1.37	0.14	0.32
Promicromonospora	0.54	1.37	0.14	0.32
Actinobaculum	0.00	1.37	0.14	0.43
Actinomyces	0.00	1.27	0.76	0.64
Parvopolyspora	0.00	1.37	0.14	0.43
Planobispora	0.00	1.27	0.76	1.04
Sanguibacter	0.00	1.37	0.14	0.32
Streptomonospora	0.00	1.37	0.14	0.32
Williamsia	0.00	1.37	0.14	0.32

indigenous ectorhizosphere population, but the effect of microbial inoculants on the endophyte population was unknown. This study used T-RFLP analysis to assess the effect of a mixed microbial soil inoculant and single endophytic actinobacterial seed inoculants on the indigenous endophytic actinobacterial population in the roots of wheat.

The T-RFLP technique was first assessed for the ability to detect a single actinobacterial endophyte introduced to the roots of wheat by seed-coating application. The specific TRF peaks corresponding to *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *N. albus* EN46 digested with HinfI, HhaI, and MboI were used to monitor colonization levels in wheat roots. The results indicated that *Microbispora* sp. strain EN2 and *Streptomyces* sp. strain EN27 colonized the wheat root from the increase in peak area corresponding to the specific fragments. Colonization of *N. albus* EN46 appeared to occur only in one of the three replicate plants, as the specific peak areas corresponding to this strain decreased for the HinfI and HhaI fragments and increased significantly for the MboI fragment for one of the replicates.

The endophytic actinobacteria *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *N. albus* EN46 were all previously isolated from wheat roots (6). A previous study by Coombs and Franco (7) showed these isolates were all capable of significant suppression of disease symptoms caused by *G. graminis* var *tritici* at 4 weeks. *Streptomyces* sp. strain EN27 also

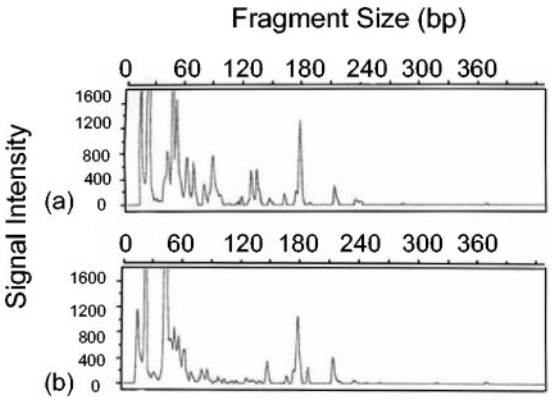

FIG. 2. T-RFLP profile for the roots of wheat grown for 6 weeks in field soil from Swedes Flat without NutriLife 4/20 (a) and with NutriLife 4/20 (b) and digested with HinfI.

showed significant growth regulation of wheat at 4 weeks. The T-RFLP results have shown that at 6 weeks the introduced endophytes increase in colonization level by up to threefold, suggesting that the introduced endophytes do not need to colonize at high levels to exhibit a positive effect on the wheat plant. This is of importance in relation to the use of endophytes as biological control agents.

As the T-RFLP technique was able to detect the introduced endophytes, the technique was used again to determine whether the indigenous endophytic actinobacterial population changes in response to the introduction of a single actinobacterial endophyte. The results indicated that the introduction of an endophyte to wheat roots did not disrupt the indigenous endophytic actinobacterial microflora. The T-RFLP technique detected 58 actinobacterial genera, with 49 genera having a maximum abundance percentage of more than 0.6%, with approximately 92% of the genera detected present in at least three of the treatments. The predominant genera in all treat-

TABLE 5. 16S rRNA gene sequence TRFs (sizes in base pairs) obtained with HinfI, HhaI, and MboI for the roots of wheat grown for 6 weeks in field soils obtained from Swedes Flat with and without NutriLife 4/20 (n = 6)

HinfI	Without NutriLife 4/20	With NutriLife 4/20	HhaI	Without NutriLife 4/20	With NutriLife 4/20	MboI	Without NutriLife 4/20	With NutriLife 4/20
35	+	+	36	+	−	34	+	+
39	+	+	39	−	+	39	+	+
41	+	−	41	+	−	43	+	+
44	+	+	44	+	+	49	+	+
49	−	−	49	+	+	53	+	+
53	+	+	53	+	+	57	−	+
57	−	+	58	−	−	63	+	+
61	−	−	61	−	−	70	+	+
63	+	+	63	+	+	72	−	+
70	+	+	70	+	+	80	−	+
80	+	+	81	+	+	82	+	+
82	+	+	85	+	+	89	+	+
84	+	+	89	+	+	91	−	−
89	+	+	91	−	−	95	+	+
92	−	−	94	+	+	99	−	+
94	+	+	97	−	+	105	−	−
97	−	+	99	−	+	113	−	−
99	−	+	106	−	+	118	+	−
105	−	−	113	−	−	128	+	−
112	−	−	120	−	−	133	+	+
128	+	+	127	+	−	147	−	+
133	+	+	133	+	+	156	+	−
147	+	+	147	+	+	158	+	−
167	−	+	175	−	−	162	+	+
174	+	+	178	+	+	166	−	+
176	+	+	190	−	−	173	+	+
178	+	+	213	+	+	175	−	−
180	+	+	227	−	−	178	−	+
188	−	+	239	−	+	214	−	−
213	+	+	258	−	−	258	−	−
236	+	+	279	−	−			
258	−	−	321	−	−			
279	−	−	348	−	−			
284	−	−	368	−	−			
321	−	−	372	−	−			
348	−	−	385	+	+			
369	−	−	387	+	+			
407	−	−	410	+	+			
411	−	−	412	+	+			
			414	+	+			
			416	−	−			
			418	+	−			
			420	−	+			

TABLE 6. Maximum abundance percentage for each genus identified by T-RFLP in the roots of wheat grown in soil obtained from Swedes Flat with and without NutriLife 4/20 ($n = 6$)

Genus	% With NutriLife 4/20	% Without NutriLife 4/20	Decrease (%) of genera in soil with NutriLife 4/20
Mycobacterium	9.75	21.02	53.62
Bifidobacterium	2.82	20.78	86.43
Rhodococcus	9.49	20.64	54.02
Streptomyces	9.15	18.53	50.62
Nocardia	3.25	16.75	80.60
Geodermatophilus	6.24	13.76	54.65
Saccharomonospora	0.00	9.98	100.00
Arthrobacter	0.00	7.52	100.00
Microbacterium	6.24	7.41	15.79
Frankia	0.00	7.04	100.00
Gordonia	4.16	6.88	39.53
Saccharothrix	0.00	6.44	100.00
Brevibacterium	0.00	6.05	100.00
Thermonospora	6.39	5.47	−16.82
Kitasatospora	0.00	4.99	100.00
Pimelobacter	2.08	4.99	58.32
Lentzea	0.00	4.99	100.00
Agromyces	2.08	4.84	57.02
Actinomyces	0.00	4.72	100.00
Williamsia	0.00	4.46	100.00
Thermocrispum	0.00	4.46	100.00
Sanguibacter	0.00	4.46	100.00
Rubrobacter	2.08	4.46	53.36
Corynebacterium	2.08	3.48	40.23
Micrococcus	2.08	2.95	29.49
Micromonospora	0.00	2.51	100.00
Leifsonia	1.17	2.42	51.65
Dietzia	2.08	2.42	14.05
Curtobacterium	1.17	2.42	51.65
Brachybacterium	1.17	2.42	51.65
Kineococcus-like bacterium	0.00	2.07	100.00
Actinosynnema	0.00	2.00	100.00
Actinoplanes	0.00	2.00	100.00
Catellatospora	0.00	1.59	100.00
Sarraceniospora	0.00	1.06	100.00
Nocardioides	2.82	1.06	−166.04
Lechevalieria	0.00	1.06	100.00
Kribella	15.69	1.06	−1380.19
Streptoalloteichus	0.00	1.01	100.00
Spirilliplanes	0.00	1.01	100.00
Planomonospora	2.82	0.00	−100.00

ments were *Kribbella*, *Streptomyces*, *Bifidobacterium*, *Arthrobacter*, *Nocardia*, and *Rhodococcus*. *Kribbella* was detected in the noninoculated control at 13.72 and 13.21% in *Microbispora* sp. strain EN2-inoculated plants and at 13.68% in the *Streptomyces* sp. strain EN27-inoculated plants. However, in the *N. albus* EN46-treated plant, *Kribbella* species decreased to 7.19%. The *Streptomyces* species increased from 12.15% (noninoculated) to 20.12% in the *Streptomyces* sp. strain EN27-inoculated plants. However, the *Streptomyces* species also increased in the *Microbispora* sp. EN2 (18.37%)- and *N. albus* EN46-treated (30.66%) roots. Nevertheless, the results indicated the endophytic actinobacterial population was relatively stable despite the inoculation of an endophyte. This is in contrast to the results obtained when a mixed microbial inoculant was added to the soil in which the wheat was cultivated.

The T-RFLP method detected 40 actinobacterial genera present in the roots of wheat grown in Swedes Flat soil without the added microbial inoculant. When NutriLife 4/20 was added, both the endophytic actinobacterial population and the relative level of colonization were reduced by approximately half. The actinobacterial genera detected was reduced to 21 genera, and the relative level of colonization of individual genera decreased to a range of 14 to 86%. There were three genera for which the maximum abundance percentages increased in soil with the added inoculant. *Kribbella* species increased from 1.06 to 15.69%, *Thermomonospora* spp. increased from 5.47 to 6.39%, and *Nocardioides* spp. increased from 1.06 to 2.82%. These actinobacterial genera were not components of the inoculant, hence their presence may be site specific or they may posses a higher level of colonization competitiveness compared to that of other indigenous microflora. Even though the mixed inoculant contained *S. albidoflavus* and *S. cellulosae*, the abundance percentage of endophytic *Streptomyces* spp. decreased from 18.53 to 9.15%. Not all soil microbes are expected to have the capability of endophytic colonization, and *S. cellulosae* and *S. albidoflavus* have not been reported to have an endophytic association with plants.

These results suggest the microbes present in the NutriLife 4/20 inoculum are able to outcompete the indigenous actinobacterial microflora, with the exception of *Kribbella*, *Nocardioides*, and *Thermomonospora*. The presence of the mixed commercial inoculant appears to prevent a number of actinobacterial genera from access to the germinating seed, leading to a reduction of endophytic colonization of the wheat roots. Many biocontrol strains are capable of improving plant growth and disease resistance in controlled pot trials but have been unsuccessful when taken out into the field. This has been attributed mainly to the inability to compete in the complex rhizosphere environment. It is possible that the addition of biocontrol strains to the soil causes disruption of the natural endophyte population, which in turn may have a negative effect on plant growth and disease resistance. Of the endophytic actinobacteria isolated from the wheat roots by Coombs and Franco (6), a number were capable of causing significant disease resistance and growth promotion (7), indicating that indigenous actinobacterial endophytes have a role in maintaining plant health.

The conclusions from this study are that reintroduction of an endophyte to a wheat seed does not significantly affect the indigenous endophytic actinobacterial population. In contrast, the addition of a mixed microbial inoculant to the soil reduces the endophytic actinobacterial diversity and relative level of colonization. This knowledge is of importance because endophytes have a role in maintaining plant health.

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REFERENCES

1. **Araujo, W. L., J. Marcon, W. Maccheroni, J. D. van Elsas, J. W. L. van Vuurde, and J. L. Azevedo.** 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. **68:**4906–4914.
2. **Araujo, W. L., W. Maccheroni, C. I. Aguilar-Vidoso, and P. A. V. Barroso.** 2001. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can. J. Microbiol. **47:**229–236.
3. **Bloemberg, G. V., and B. J. Lugtenberg.** 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4:**343–350.

4. **Brooks, D. S., C. F. Gonzalez, D. N. Appel, and T. J. Filer.** 1994. Evaluation of endophytic bacteria as potential biological control agents for oak wilt. Biol. Control **4:**373–381.
5. **Conn, V. M., and C. M. M. Franco.** 2004. Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism (T-RFLP) and sequencing of 16S rRNA clones. Appl. Environ. Microbiol. **70:**1787–1794.
6. **Coombs, J. T., and C. M. M. Franco.** 2003. Isolation and identification of actinobacteria isolated from surface-sterilized wheat roots. Appl. Environ. Microbiol. **69:**5303–5308.
7. **Coombs, J. T., P. P. Michelsen, and C. M. M. Franco.** 2003. Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis var. tritici* in wheat. Biol. Control **29:**359–366.
8. **Garbeva, P., L. S. Overbeek, J. W. van Vuurde, and J. D. van Elsas.** 2001. Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microb. Ecol. **41:**369–383.
9. **Germida, J. J., S. D. Siciliano, R. de Freitas, and A. M. Seib.** 1998. Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol. Ecol. **26:**43–50.
10. **Heuer, H., M. Krsek, P. Baker, K. Smalla, and E. M. H. Wellington.** 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. **63:**3233–3241.
11. **Hoisington, D., M. Khairallah, T. Reeves, J. M. Ribaut, B. Skovmand, S. Taba, and M. Warburton.** 1999. Plant genetic resources: what can they contribute toward increased crop productivity? Proc. Natl. Acad. Sci. USA **96:**5937–5943.
12. **Hoitink, H., and M. Boehm.** 1999. Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. Annu. Rev. Phytopathol. **37:**427–446.
13. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood (ed.).** 2000. Practical Streptomyces genetics. John Innes Centre, Norwich, England.
14. **Kloepper, J. W., R. Rodriguez-Kabana, G. W. Zehnder, J. Murphy, E. Sikora, and C. Fernandez.** 1999. Plant root-bacterial interactions in biological control of soilborne disease and potential extension to systemic and foliar disease. Aust. Plant Pathol. **28:**27–33.
15. **Lukow, T., P. F. Dunfield, and W. Liesack.** 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol. Ecol. **32:**241–247.
16. **Marsh, T. L., P. Saxman, J. Cole, and J. Tiedje.** 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl. Environ. Microbiol. **66:**3616–3620.
17. **McInroy, J. A., and J. W. Kloepper.** 1995. Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can. J. Microbiol. **41:**895–901.
18. **M'Piga, P., R. R. Belanger, T. C. Paulitz, and N. Benhamou.** 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63–28. Physiol. Plant Mol. Biol. **50:**301–320.
19. **Nejad, P., and P. A. Johnson.** 2000. Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. Biol. Control **18:**208–215.
20. **Provorov, N. A., A. Y. Borisov, and I. A. Tikhonovich.** 2002. Developmental genetics and evolution of symbiotic structures in nitrogen-fixing nodules and arbuscular mycorrhiza. J. Theor. Biol. **214:**215–232.
21. **Sessitsch, A., B. Reiter, U. Pfeifer, and E. Wilhelm.** 2001. Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiol. Ecol. **1305:**1–10.
22. **Siciliano, S. D., C. M. Theoret, J. R. de Freitas, P. J. Huci, and J. J. Germida.** 1998. Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. Can. J. Microbiol. **44:**844–851.
23. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67:**4742–4751.
24. **Sturz, A. V., B. R. Christie, B. G. Matheson, W. J. Arsenault, and N. A. Buchanan.** 1999. Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. Plant Pathol. **48:**360–369.
25. **Sturz, A. V., B. R. Christie, and B. G. Matheson.** 1998. Associations of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. Can. J. Microbiol. **44:**162–167.
26. **Tokala, R. K., J. L. Strap, C. M. Jung, D. L. Crawford, H. Salove, L. A. Deobald, F. J. Bailey, and M. J. Morra.** 2002. Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Appl. Environ. Microbiol. **68:**2161–2171.
27. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
28. **Whipps, J. M.** 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. **52:**487–511.
29. **Xaio, K., L. L. Kinkel, and D. A. Samac.** 2002. Biological control of *Phytophthora* root rots on alfalfa and soybean with *Streptomyces*. Biol. Control **23:**285–295.
30. **Yuan, W. M., and D. L. Crawford.** 1995. Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. Appl. Environ. Microbiol. **61:**3119–3128.